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TITLE: IGG SUBCLASS & ISOTYPE SPECIFIC IMMUNOGLOBULIN  
RESPONSES TO LASSA FEVER & VENEZUELAN EQUINE  
ENCEPHALOMYELITIS: NATURAL INFECTION AND  
IMMUNIZATION

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<p>Venezuelan Equine Encephalitis (VEE) specific Immunoglobulin responses to the two vaccines TC83 (a live-attenuated vaccine) and C-84 (a Formalin inactivated vaccine derived from the TC-83 strain) were evaluated using an antigen and isotype specific enzyme-linked immunoabsorbant assay (ELISA). The VEE specific ELISA was developed and standardized using selected sera from individuals previously immunized. Initial experiments demonstrated that non-specific binding in the IgG, IgG2, IgA, IgM assays was a significant problem in data interpretation. Preparations of a negative control (Tissue Culture Media derived) antigen were utilized to further standardize the assays.</p> <p>All assays were standardized in methodology and in relation to a uniform reference curve where available. Initial screening of 28 sera demonstrated the presence of predominantly VEE-Specific G1 and G3 subclasses, no G4 and erratic, predominantly low level G2. IgA and IgM responses were also demonstrated in the sera of subjects who received the TC-83 vaccine</p>					
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as well as in the response to a C-84 booster vaccine.

Lassa Fever specific antibody assay development was delayed by the lack of availability of the antigen. Keywords: Toga Virus, Arenavirus,

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In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

RM For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Renata J. M. Engler 11 NOV 88  
PI Signature Date

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## INTRODUCTION AND BACKGROUND

Immunoglobulin responses to a wide range of infectious agents have been associated with protective immunity, either short or long term. However, in the case of viral infections, virus-specific antibody measurements are not consistently correlated with in vitro neutralization potency or with effective natural immunity. These observed variations may in some cases be due to differences in viral antigens recognized, but they may also reflect differences in isotype or IgG subclass responses that have different efficiency in mediating effective immunity.

Antigen-specific IgG production is generally associated with long-term immunity to a wide range of bacterial and viral infectious agents.(1,2) Human IgG is subdivided into 4 subclasses, each having distinct biologic properties and functions.(1)

VEE, an arthropod-borne RNA virus representative of the alpha-viruses in the Togaviridae group, produces epidemic and endemic disease in Central and South America as well as the southern United States. The equine population serves as the principal viremic host but rodents and marsupials can also harbor the virus. Morbidity associated with this disease is considerable but mortality in adults is low (perhaps less than 1%). In children however, the case fatality rate with encephalitis is as high as 35 %. The infection/viremia generates a brisk and promptly effective (neutralizing) antibody response in the host and hyperimmune serum provides effective passive

immunity.(3) The IgG subclass responses to natural infection and vaccination have not been well described for the Venezuelan Equine Encephalomyelitis virus (VEE).

Two types of vaccines are currently available for the prevention of VEE in man and horses.(4) TC-83, a live attenuated vaccine produced by serial passage of the wild virus utilizing guinea pig fetal heart cell culture, has proved to be efficacious (providing long term immunity) and relatively safe for immunizing horses and man. However, up to 25 percent of individuals vaccinated develop clinical illness with a low grade viremia.(5) In addition, this vaccine may have abortogenic and teratogenic potential and is relatively ineffective in boosting marginal antibody responses.(6)

C-84 is a formalin-inactivated vaccine derived from the TC-83 strain of virus which has been shown to be safe and effective in inducing serum antibody.(4,6) This vaccine produces only mild local and systemic reactions and induces a high titer of neutralizing antibody in both non-immune subjects after 3 immunizations and in sero-positive TC-83 recipients (positive booster effect).(6,7) This vaccine provides effective protection for experimental animals infected by virulent VEE strains by injection but not by aerosol. The mechanism for this is not understood. Vaccinated humans have not been exposed to virulent virus to permit any conclusions about protective efficacy in man.

Virus specific IgG subclass responses to vaccines may vary with vaccine type and may play a role in the difference in protective potential between

the inactivated versus live attenuated vaccines. Selective stimulation of certain subclasses of antibody may be more important in the future when adjuvants and smaller antigens may be used for immunizing agents. In addition, the determination of the IgG subclass predominantly associated with neutralizing antibody could be useful if serum or monoclonal antibodies were to be used for passive immunization against VEE.

The purpose of this phase of the study was to develop specific and sensitive assays for the measurement of IgG, IgG subclasses, IgA and IgM directed against VEE antigens. These assays would then be applied to the evaluation of specific antibody responses to the live attenuated (TC-83) and the formalin-inactivated vaccine (C-84). The issue of correlation to the "gold standard", viral neutralization, could then be explored for each of the isotypes and G subclasses.

#### EXPERIMENTAL METHODS

Human sera positive and negative for VEE specific antibody by viral neutralization assay were obtained through the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland. These sera had been previously collected under Human Use Committee approved informed consent including permission for utilization in other studies as long as the confidentiality of records was maintained.

VEE antigen was also prepared at the USAMRIID facility as follows:  
TC-83 strain VEE concentrate grown in BHK-21 cell line (Clone 13);  
gradient purified preparation inactivated by 6 times 10E6 rads of



cobalt irradiation. A positive pooled reference serum (with significant high titer 80% plaque reduction on viral neutralization assay) was prepared and utilized for initial testing. Optimum coating concentrations and conditions were tested and subsequently fixed as follows:

1. Dynatech Immulon-2-flat microelisa plates (011-010-3650) were coated with VEE antigen using a carbonate buffer at Ph 9.6 overnight at 4 degrees centigrade in a humidified chamber at 37 degrees centrigade. Plates can be stored coated without loss of activity for at least a one week period. Each new batch of VEE antigen must be tested with the reference serum in order to decrease inter-assay variability with changes in antigen lots. In addition, storage of aliquoted antigen frozen at minus 70 degrees C. longer than 6 months results in some decay of specific binding at a fixed coating concentration. Coating concentrations varied from a 1:150 dilution to a 1:400 dilution. Assays over time were noted to remain uniform if conditions were always corrected to the same reference serum curve.
2. Multiple initial experiments with different positive and negative sera (and commercially available pooled gammaglobulin from many thousands of donors but known to have negative viral neutralization properties) demonstrated the fact that certain assays were complicated by high background binding. A VEE negative antigen derived from the same culture media as the positive antigen could be utilized to correct for the background binding which was

highly variable between individual sera. Alternate columns were subsequently coated with positive and negative antigen, and each serum was run in parallel with at least 4 dilutions on the same plate.

The basic methodology used in the performance of the ELISA's is well described in the Manual of Clinical Laboratory Immunology.(8) Specific reagents tested and utilized during the assay and special procedural considerations are outlined as follows:

1. Sera were initially tested at a dilution of 1:40 with a serial 1:2 dilution on the plate to include at least 4 dilutions. Post vaccine exposure sera were started at 1:160. Later experiments started baseline sera at 1:80 and high titer post vaccine sera at 1:320. Individual subject sera pre and post vaccination were always run in parallel on the same plate in order to decrease intra-assay variability and optimize titer-fold reproducibility (post divided by pre vaccine titers). Sera were diluted in phosphate buffered saline (PBS) with 0.05% Tween-20 and 0.1% bovine serum albumin (0.02% azide as preservative) (PBS-Tween-BSA) and incubated overnight.
2. Detection of specific isotypes and subclasses utilized reagents tested in multiple experiments for specificity (using purified myeloma proteins) and sensitivity. Final selection of the following reagents for the detection antibody included the following:  
  
(NOTE: all reagents were diluted in PBS-Tween-BSA and incubated on the plate at least 90 minutes at 37 degrees C.)

- a. Affinity-purified goat anti-human IgG, alkaline phosphatase conjugated (TAGO, Inc., Palo Alto, CA, catalogue no. 4300)
- b. Affinity-purified goat anti-human IgM, alkaline phosphatase (AP) conjugated (TAGO catalogue number 4302)
- c. Affinity-purified goat anti-human IgA, fluorescein isothiocyanate (FITC) conjugated (TAGO catalogue number 4201) followed by an affinity-purified goat anti-FITC, AP conjugated (special order RD009 from TAGO, Inc.).
- d. Mouse monoclonal antibodies specific for human IgG subclasses were selected for specificity following testing with a panel of purified G subclass myeloma proteins. The selected antisera are listed below and are currently recognized by the World Health Organization as specific for human IgG subclasses:  
  
\* G-1 (HP 6001); G-2 (HP 6014); G-3 (HP 6050); G-4 (HP 6025).  
  
These reagents were graciously provided by Dr. Reimer of the Center for Disease Control, Atlanta, GA.

3. Subsequent reagents utilized in the G subclass assays included the following:

- a. Affinity-purified goat anti-mouse-FITC conjugated (Coulter catalogue number 6602159) followed by the previously listed anti-FITC.

The intermediate steps between reagents and the final development step with the substrate, p-nitrophenylphosphate (Sigma Chemicals), are well described previously.(8)

Plates were read utilizing an MR-600 Dynatech Microflour reader utilized in conjunction with an Apple IIe computer and the software Immunosoft version 2.4. VEE antigen negative (VEE -) optical densities were subtracted from VEE positive (VEE +) antigen binding in parallel dilutions. The negative binding was significantly above plate/reagent background for the IgG, IgA, IgM, and IgG-2 assays but not for G-1 or G-3 (or G-4). Each assay was standardized to the uniform reference curve and both end-point titers and units/ml (based on assigned units to the reference curve) were calculated using log-logit transformations and curve fitting.(9)

Viral neutralization assays specific for VEE are performed routinely at the USAMRIID laboratories and 80% plaque reduction/conversion titers for each of the sera studied were provided courtesy of J. Mangiofico.

#### CONCLUSIONS

ELISA studies for the measurement of VEE specific antibody have resulted in the following findings:

1. VEE specific IgG is present in high titers in pooled sera from subjects who have received TC-83 vaccine alone or C-84 booster vaccination. Appendix 1 is a graph of the optical density readings versus the log of the inverse titer for the VEE pooled reference (#2) against positive VEE antigen and negative control antigen. The VEE negative antigen binding is particularly significant in the lower serum dilutions. Also illustrated is the serial dilution binding of a single lot of commercially available GAMMAGARD (GG) and of SANDOGLOBULIN (SANDO). These two products contain primarily IgG

from more than a thousand normal donors and are essentially negative for VEE specific viral neutralization (but do have low level activity to cross-reactive Western and Eastern Equine Encephalitis viruses). The negative antigen binding control is essential for assay interpretation and elimination of false positive assays. Appendix #2 is a representative curve of IgG binding from GAMMAGARD to VEE - and + antigens.

2. VEE-specific IgG-1 is present in significant amounts in sera with positive viral neutralization. Background binding to VEE - antigen is in low levels and not significantly different from plate/reagent background binding. The same is true for VEE-specific G-3 and Appendix #3 illustrates a representative VEE-specific G-1 and G-3 reference curve (after VEE - binding correction).
3. VEE-specific G-2 is present in low levels in the pooled reference serum even after correction for significant VEE - antigen binding. Initial testing of individual sera before and after immunization suggests erratic responses not correlated to viral neutralization titers. Appendix #4 illustrates a representative VEE reference G-2 curve for both positive and negative antigen binding.
4. VEE-specific IgG titer increases correlate best with G-1 increases as illustrated in Appendix #5 (G titer increase versus G-1).
5. There is no VEE specific G-4 demonstrated in any of the sera tested to date.
6. VEE specific IgM was present in the pooled reference but with

significant binding also demonstrated to the negative antigen.

(Appendix #6)

7. VEE specific IgA was also demonstrated in the reference serum and, like IgM, negative antigen binding was present in significant amounts. However, paired sera again demonstrated significant increases after vaccination even after correction for negative binding.
8. Viral neutralization assay titers (80% plaque reduction titers) appear to correlate well to VEE-specific G, G1 and G3 titers. These tests are in progress at the present time.

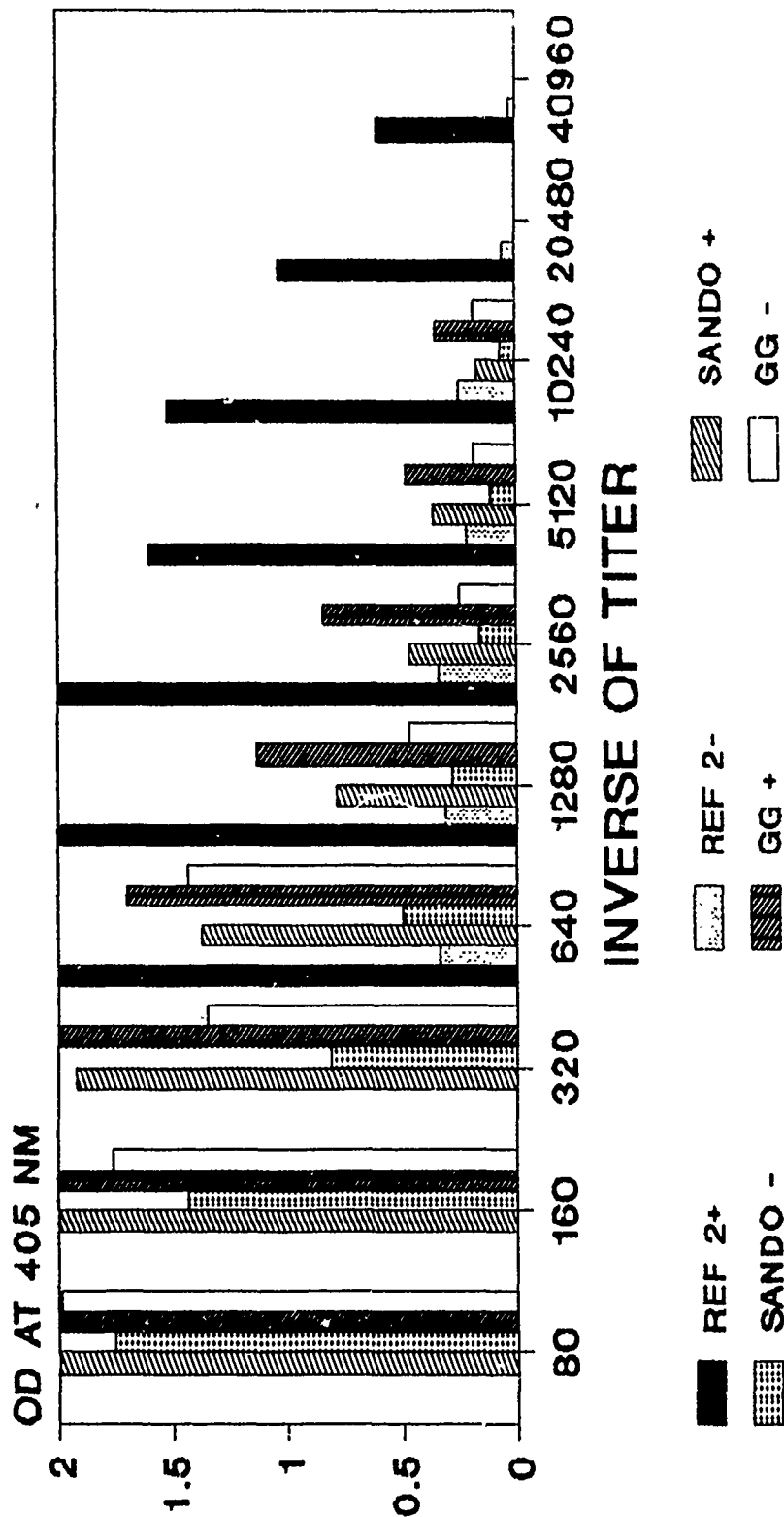
Further refinements of the VEE-specific IgA and IgM assay may be necessary in the next phase of the study. Application of the assays to different subject groups, i.e., individuals vaccinated with TC-83 alone, C-84 boosters, C-84 alone and larger negative control groups, may provide additional information regarding assay specificity, vaccine response differences and clearer correlations to the "gold standard", viral neutralization. ELISA's specific for VEE G1 and G3 responses may be useful screening tests and may be adequate substitutes for the neutralization assay which is more difficult to perform.

The next phase of the project will include the development of isotype specific ELISA's for Lassa Fever virus as outlined in the original research proposal. Lack of availability of the antigenic material has delayed initiation of the early experiments for establishing assay conditions.

## REFERENCES

- 1 Schur PH: IgG subclasses - a review. ANN ALLERGY 1987; 58(2): 89-96.
- 2 Beck OE: Distribution of virus antibody activity among human IgG sub-classes. CLIN EXP IMMUNOL 1981; 43: 626-28.
- 3 Leon CA, Jaramillo R, Martinez S, Fernandez F, Tellez H, Lasso B, Guzman R de: Sequellae of Venezuelan Equine Encephalitis in humans: a 14 year follow up. INT J EPIDEMIOL 1975; 4:131.
- 4 McKinney RW: Inactivated and live VEE vaccines - a review. VENEZUELAN ENCEPHALITIS SCIENTIFIC PUBLICATION No. 243, PAN AMERICAN HEALTH ORGANIZATION 1972; 369-89.
- 5 London NT, Levitt NH, Kent SG, Wong VG, Sever JL: Congenital cerebral and ocular malformations induced in rhesus monkeys by Venezuelan equine encephalitis virus. TERATOLOGY 1977; 16:285-96.
- 6 Edelman R, Ascher MS, Oster CN, Ramsburg HH, Cole FE, Eddy GA: Evaluation in humans of a new, inactivated vaccine for Venezuelan Equine Encephalitis Virus (C-84). J INFECT DIS 1979; 140(5): 708-715.
- 7 Cole FE, May SH, Eddy GA: Inactivated Venezuelan equine encephalomyelitis vaccine prepared from attenuated (TC-83) strain virus. APPLIED MICROBIOLOGY 1974; 27:150-3.
- 8 Voller A, Bidwell D: Enzyme-linked immunosorbent assay. MANUAL OF CLINICAL LABORATORY IMMUNOLOGY 1986; 99.
- 9 Channing Rodgers RP: Data processing of immunoassay results. MANUAL OF CLINICAL LABORATORY IMMUNOLOGY 1986 (American Society of Microbiology); 82.

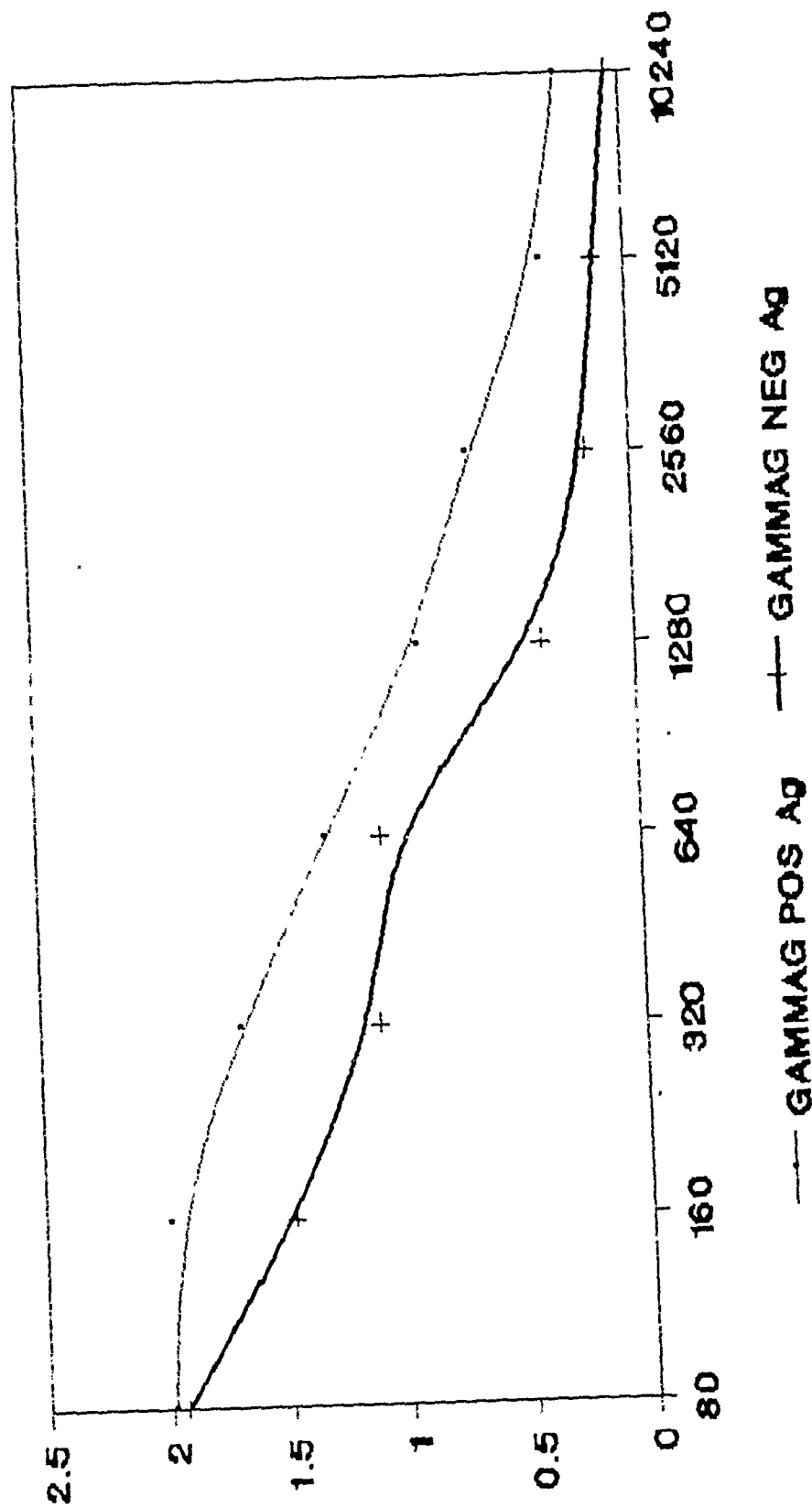
# VEE (+ & -) WITH REF #2: IgG AND SANDOGLOBULIN & GAMMAGARD



20 MINUTE READING; 3N NAOH  
COATING AT 1:400 CARB BUFFER; FEB '89

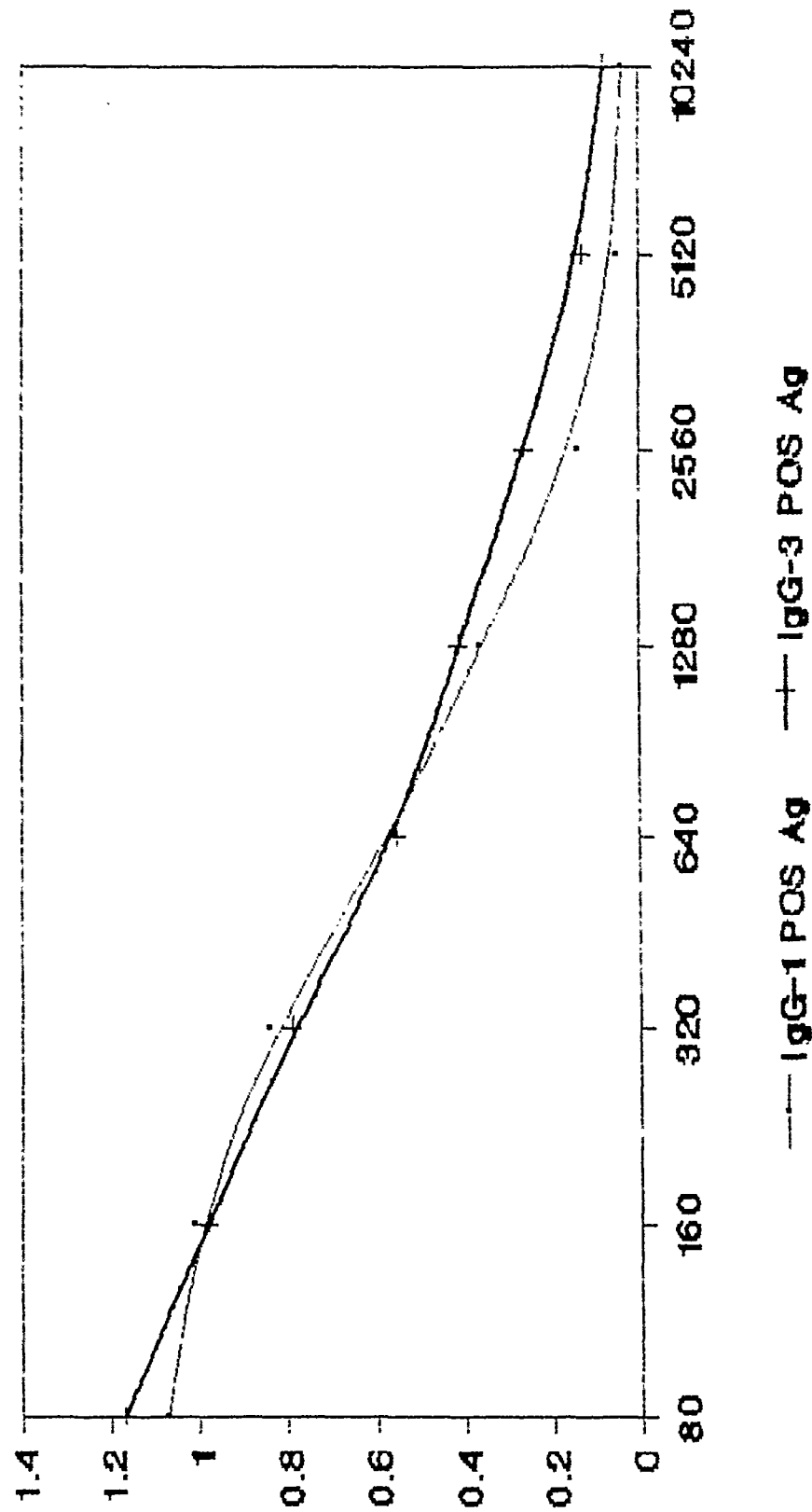


# VEE SPECIFIC IgG IN GG GAMMAG (+VS-)



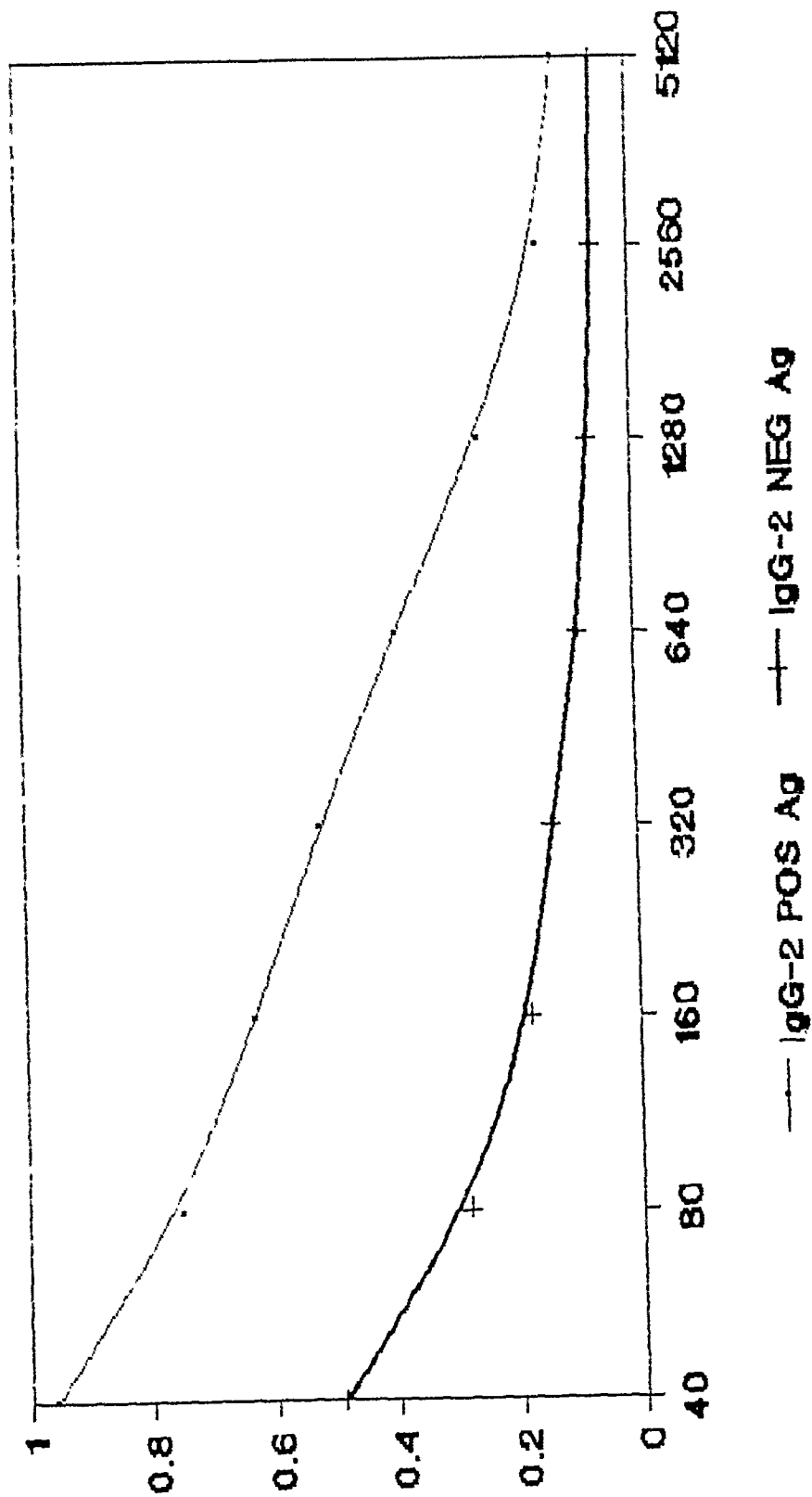
SERUM TITER VERSUS O.D. AT 405 NM

# VEE REF #: IgG-1 & IgG-3 TITER VS OD AT 405 NM



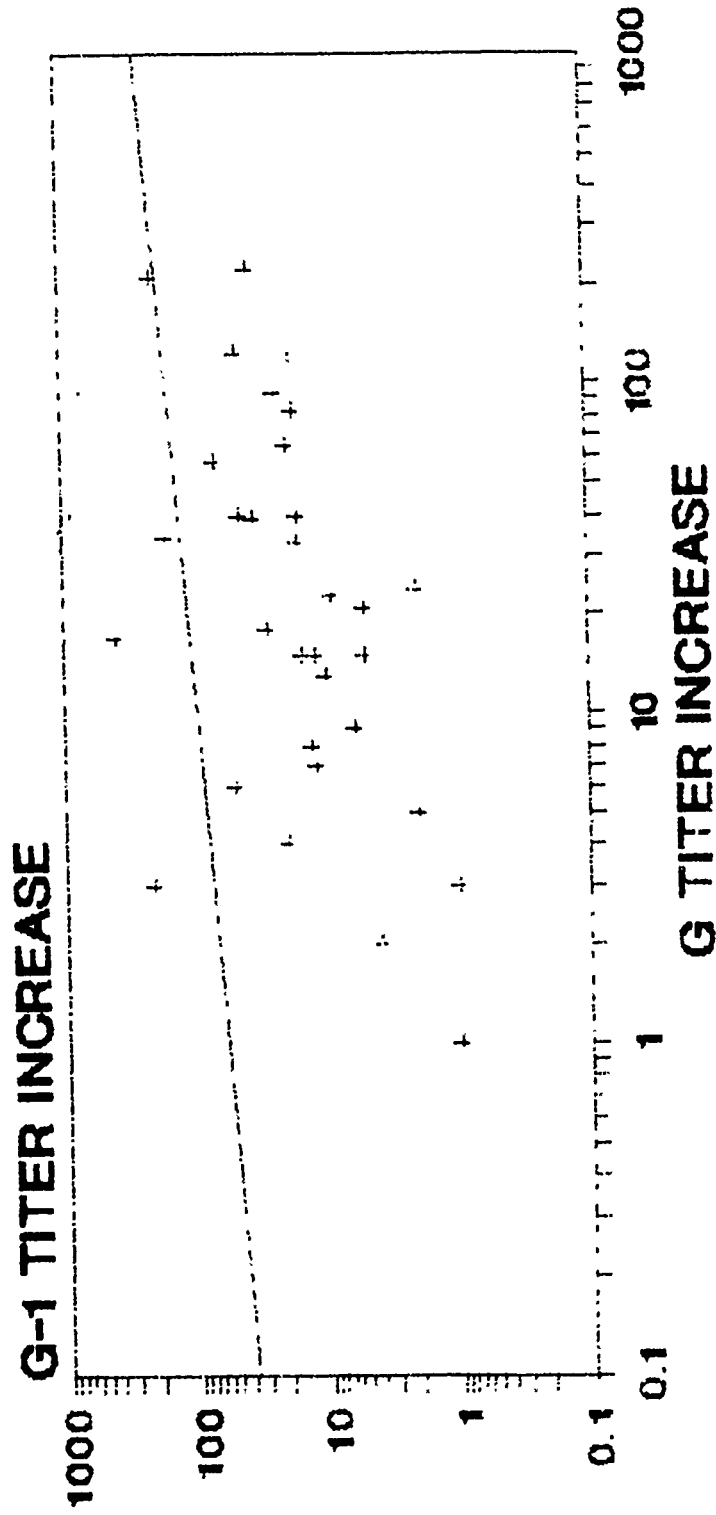
VEE NEG AG OD < 0.15 AT 1:80

# VEE REF #2: IgG2 TITER VS OD AT 405 NM



VEE POS Ag VS NEG Ag

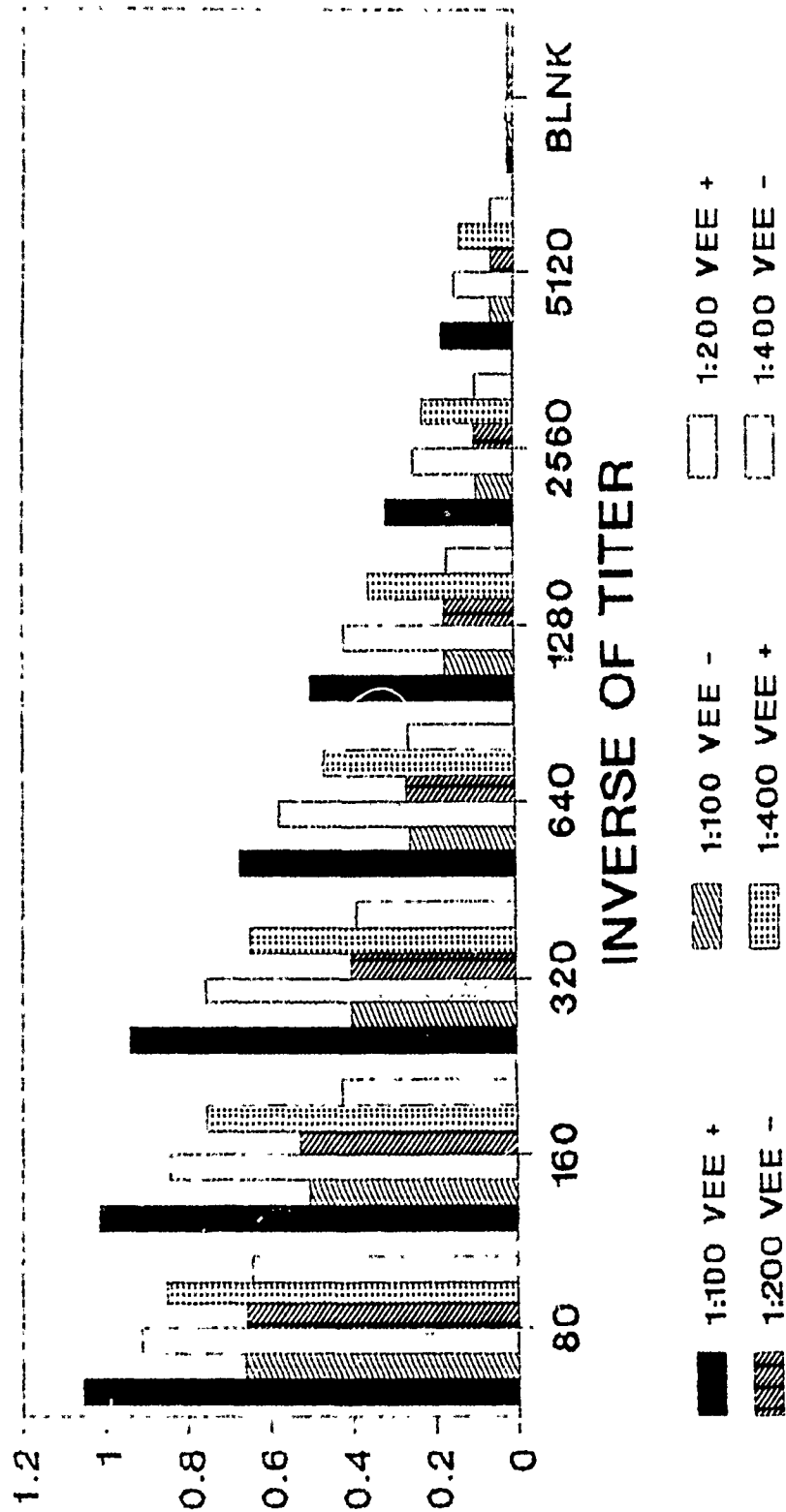
# **VEE SPECIFIC IgG VERSUS IgG-1 TITER INCREASES PRE TO POST TC-83**



--- POST TC83 TITER INCR

RATIO OF G VS G1 IN RELATIVE ELISA UNITS  
UNITS/ML COMPARED TO POOLED REF SERA-1

# VEE COATING CONCENTRATIONS: IgM POSITIVE VS NEGATIVE CONTROL AG'S



15 MINUTE READING; 3N NAOH  
OD AT 405 NM (X-AXIS)